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(54) Title: AMINOACYLASE AND ITS USE IN THE PRODUCTION OF D-AMINOACIDS

(57) Abstract

An isolated enzyme capable of hydrolysing N-acetyl-D-tryptophan at a substrate concentration of 10 g/l and which exhibits faster conversion of (R)-N-acetyl-2-thienylalanine than of (R)-N-acetyl-4-chlorophenylalanine. This enzyme is useful for preparing D-aminoacids.

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AMINOACYLASE AND ITS USE IN THE PRODUCTION OF D-AMINOACIDS

Field of the Invention

This invention relates to an enzyme having D-aminoacylase activity and to its use in the production of D-aminoacids, by resolving a racemic mixture of N-acyl aminoacids and deprotecting optically-enriched N-acyl aminoacids.

Background of the Invention

D-Aminoacids are commercially important intermediates in the production of various pesticides, antibiotics and other pharmaceuticals. For example, phenylglycine and p-hydroxyphenylglycine are used in the synthesis of semi-synthetic penicillins and cephalosporins. There is also much demand for novel D-aminoacids as building blocks for new drug substances.

D-Aminoacids may be accessed by physical separation, for example by crystallisation of salts, or by asymmetric chemocatalysis by way of hydrogenation of an enamide precursor. Chemocatalysis provides a general method of broad applicability, e.g. for unnatural aminoacids, but requires subsequent N-deacylation for which conventional chemical hydrolysis often results in partial racemisation of the product. There are biocatalytic methods also, for example by the hydrolysis of hydantoins using a D-specific hydantoinase. However, the resulting D-carbamoyl aminoacid still requires enzymic or chemical deprotection to the aminoacid.

The production of L-aminoacids by means of an L-specific aminoacylase-catalysed hydrolysis of the racemic N-acetylaminoacid is a technology that is well established. This uses the enzyme from *Aspergillus oryzae* and has been operated on a commercial basis at very large scale, to produce L-methionine, L-valine and L-phenylalanine. Such a large-scale technology does not exist for production of D-aminoacids, although D-aminoacylase activity has been identified in several microbial strains of *Pseudomonas, Streptomyces* and *Alcaligenes*. See Sugie and Suzuki, Agric. Biol. Chem. **44**:1089-1095 (1980); Daicel Chemical Industries, JP 64-5488 (1989); Moriguchi and Ideta, Appl. Env. Microbiol. **54**: 2767-2770 (1988); Sakai *et al*, Agric. Biol. Chem. **54**: 841-844 (1990); Sakai *et al*, J. Ferm. Bioeng. **71**:79-82 (1991); Sakai *et al*, Appl. Env. Microbiol. **57**: 2540-2543 (1991); Yang *et al*, Appl. Env. Microbiol. **57**: 1259-1260 (1991); and Kameda *et al*, Nature **169**: 1016 (1952).

The enzymes from these strains were isolated and characterised. It should in theory be relatively easy to use such strains in whole-cell form for the resolution or deprotection of *N*-acetyl aminoacids, but the cells were also shown to contain L-aminoacylases, thus reducing the stereoselectivity. In addition, the low levels of activity, even after growth on inducing media, make purification and use of the enzyme from the whole-cell unattractive economically. A solution was foreseen by way of cloning the enzymes, and this has been reported recently for an *Alcaligenes* species D-aminoacylase, though it would not be expected that such an enzyme would work at any higher substrate concentration, nor differ significantly in its substrate specificity from the wild-type enzyme. See Moriguchi *et al*, Biosci. Biotech. Biochem. **57 (7)**:1149-1152 (1993); Wakayama *et al*, Biosci. Biotech. Biochem. **59 (11)**: 2115-2119 (1995); and Wakayama *et al*, Prot. Express. Pur. 7: 395-399 (1996).

This enzyme, obtained from *Alcaligenes xylosoxydans* subsp. *xylosoxydans* ("*Alcaligenes* A-6"), NCIMB 10771, does not hydrolyse N-acetyl-D-tryptophan. It is reported that the activity of D-aminoacylase is inhibited by 37% and 40% by D-phenylalanine and N-acetyl-D-alloisoleucine at a very low concentration of 2mM. This suggests that the enzyme is susceptible to severe product and substrate inhibition.

US-A-5206162 discloses a D-aminoacylase obtained from *Alcaligenes faecalis*, CCRC 14817.

EP-A-0896057 (published after the first priority date claimed for this Application) discloses a D-aminoacylase obtained from *Amycolatopsis orientalis*, IFO 12806.

Summary of the Invention

The present invention was made following a screen for D-aminoacylase activity performed on a collection of bacteria, and from this screen several were identified as having a D-aminoacylase. Five of these strains were used for genomic DNA preparation. It was then possible, by examining a known literature sequence, to design oligonucleotide primers, and use these in PCR experiments to generate a 1.4kb fragment possessing D-aminoacylase activity. The recombinant fragment was sub-cloned into pTrc99C expression vector. The recombinant plasmid carrying the D-amino acylase fragment was then transformed in to *E. coli* DH5 for over-expression. After fermentation of the host, cells were obtained which had good D-aminoacylase activity.

Sequencing of the enzyme showed that it had six differences to the published sequence of the known cloned *Alcaligenes* D-aminoacylase. These were Ser^{2} to Ala; Gln^{3} to Glu; Ala^{14} to Val; Gly^{126} to Arg; Gly^{240} to Arg; and Glu^{242} to Lys. These differences, individually or in combination, apparently bring about notable and surprising differences in the properties of the enzyme. For example, the novel enzyme will hydrolyse N-acetyl-D-tryptophan, whereas the published enzyme does not. Surprisingly, this enzyme is active at high substrate concentration; the published literature gives only examples of low substrate concentration, in the region of 20mM. At these concentrations, the volume efficiency is low, which increases the cost of recovering the product and reduces the economic viability of the process. Thus, it was surprising to find that the enzyme is effective at 100g/l of substrate; even at 200g/l good activity was demonstrated. It is useful at high volume efficiency, of about 100g/l, for the deprotection of several (D)-N-acetylaminoacids. This allows an economical process to be developed.

More generally, an isolated enzyme according to the present invention is capable of hydrolysing N-acetyl-D-tryptophan at a substrate concentration of 10 g/l. Thus, it is capable of the desired activity at the given concentration, and also at higher concentrations. In addition, unlike the enzymes disclosed in US-A-5206162 and in EP-A-0896057, it exhibits the ability to convert (R)-N-acetyl-2-thienylalanine, and also to convert it faster than (R)-N-acetyl-4-chlorophenylalanine.

Description of the Invention

In general terms, the substrate used in the invention may be part of a mixture of the (L)- and (D)-N-acylaminoacids. Alternatively, the (D)-N-acylaminoacid may be enantiomerically enriched, e.g. essentially optically pure.

The novel enzyme may be used to produce natural and unnatural aminoacids. One class of the latter is aryl/heteroaryl-substituted aminoacids.

In some instances, particularly with substrates of a hydrophobic nature, the enzyme may suffer from substrate inhibition. In these cases, for example with N-acetyl-D-styrylalanine or N-acetyl-D-2-naphthylalanine, a high substrate concentration may merely lead to a low conversion to product, so that a volume efficient reaction is not possible. However, this effect can be overcome by the simple expedient of adding the substrate in several batches over the course of the biotransformation, and, if kept at low concentration, a high product accumulation is possible. For example, if the enzyme is exposed to >20g/l

of N-acetyl-D-2-naphthylalanine, substrate hydrolysis is poor. However, the enzyme will hydrolyse 15 g/l efficiently and, by making several additions of the substrate, it is possible to accumulate about 75 g/l of D-2-naphthylalanine.

The enzyme may be used in whole cell or isolated form. It may be immobilised, if desired, by methods known to those of ordinary skill in the art.

The enzyme may be produced from the deposited organism (details given below). Alternatively, it may be produced by recombinant technology.

Using the DNA and amino-acid sequence provided herein, a person skilled in the art can readily construct fragments or mutations of the genes and enzymes disclosed herein. These fragments and mutations, which retain the activity of the exemplified enzyme, are within the scope of the present invention. Also, because of the redundancy of the genetic code, a variety of different DNA sequences can encode the amino-acid sequences disclosed herein. It is well within the skill of one of ordinary skill in the art to create these alternative DNA sequences encoding the same, or similar, enzymes. These DNA sequences are within the scope of the present invention. As used herein, reference to "essentially the same" sequence refers to sequences which have amino-acid substitutions, deletions, additions or insertions which do not materially affect activity. Fragments retaining activity are also included in this definition.

The genes of this invention can be isolated by known procedures and can be introduced into a wide variety of microbial hosts. Expression of the gene results, directly or indirectly, in the intracellular production and maintenance of the enzyme. The gene may be introduced *via* a suitable vector into a microbial host.

A wide variety of ways are available for introducing the gene into the microorganism host under conditions which allow for stable maintenance and expression of the gene. A DNA construct may include the transcriptional and translational regulatory signals for expression of the gene, the gene under their regulatory control and a DNA sequence homologous with a sequence in the host organism, whereby integration will occur, and/or a replication system which is functional in the host, whereby integration or stable maintenance will occur.

In the direction of transcription, namely in the 5' to 3' direction of the coding or sense sequence, the construct can involve the transcriptional regulatory region, if any, and the promoter, where the regulatory region may be either 5' or 3' of the promoter, the

ribosomal binding site, the initiation codon, the structural gene having an open reading frame in phase with the initiation codon, the stop codon(s), the polyadenylation signal sequence, if any, and the terminator region. This sequence as a double strand may be used by itself for transformation of a microorganism host, but will usually be included with a DNA sequence involving a marker.

The gene can be introduced between the transcriptional/translational initiation and termination regions, so as to be under the regulatory control of the initiation region. This construct can be included in a plasmid, which could include at least one replication system, but may include more than one, where one replication system is employed for cloning during the development of the plasmid and the second replication system is necessary for functioning in the ultimate host. In addition, one or more markers may be present, as described above. Where integration is desired, the plasmid will desirably include a sequence homologous with the host genome.

The transformants can be isolated in accordance with conventional ways, usually employing a selection technique, which allows for selection of the desired organism as against unmodified organisms or transferring organisms, when present. The transformants then can be tested for activity.

Suitable host cells include prokaryotes and eukaryotes. An example is *E. coli*.

The following Examples illustrate the invention.

Example 1 **Production of D-Aminoacylase**

Genomic DNA was prepared from 5 *Alcaligenes* strains held in the Chirotech culture collection; CMC3352, 3353, 2916, 3378, 3823. From these genomic preparations PCR was carried out to amplify the D-aminoacylase reported by Wakayama *et al* (1995), supra. Primers were synthesised according to the published sequence of the *dan* gene from *Alcaligenes* A-6. The 5' PCR primer in SEQ ID NO. 1; the 3' PCR primer is SEQ ID NO. 2.

A 1.4 kb PCR fragment was amplified from strains CMC 3352 and 3353. These fragments were then cloned into the PCR cloning vector from Stratagene, pCR-script and transformed into *E. coli*. Resultant clones were analysed by restriction mapping to ascertain the presence of a 1.4kb acylase fragment. Clones harbouring this fragment were sequenced to verify that the putative acylase showed homology to the reported sequence. DNA sequence analysis show the majority of cloned fragments to include SEQ ID NO. 3.

The deduced aminoacid sequence is given below as SEQ ID NO. 4. The residues of the recombinant D-acylase differ from the published sequence as follows; Ser^{2} to Ala; Gln^{3} to Glu; Ala^{14} to Val; ; Gly^{126} to Arg; Gly^{240} to Arg; Glu^{242} to Lys.

The recombinant fragment was sub-cloned into pTrc99C expression vector via the 5' *Nco*I and 3' *Bam*HI engineered restriction sites. The recombinant plasmid carrying the D-amino acylase fragment was transformed into *E. coli* DH5 for over-expression.

The recombinant cells, *E. coli* strain CMC 4406, have been deposited at NCIMB, 23 St. Machar Drive, Aberdeen AB24 3RY, Scotland. The accession number is NCIMB 40965.

The recombinant cells were grown by fermentation in a medium containing glucose, peptides and salts. The seed culture was inoculated from plates, and incubated overnight in TSB medium containing 0.1 g/l ampicillin at 37 °C.

The inoculum (5ml, OD 5.0) was grown in 1.5l of the following medium which contained (amounts in $g.l^{-1}$ unless otherwise indicated):

KH_2PO_4	8
K_2HPO_4	7
$(NH_4)_2SO_4$	1
$MgSO_4.7H_2O$	1
Yeast Extract	15
Trace elements solution	$1ml.l^{-1}$
Glucose	10
Polypropylene glycol	$1ml.l^{-1}$
Hycase SF	15

The trace elements solution consisted of (amounts in $g.l^{-1}$ unless otherwise indicated):

$CaCl_2.2H_2O$	3.6
$CoCl_2.6H_2O$	2.4
$CuCl_2.2H_2O$	0.85
$FeCl_3.6H_2O$	5.4
H_3BO_4	0.3
HCl	$333ml.l^{-1}$
$MnCl_2.4H_2O$	2.0
$Na_2MoO_4.2H_2O$	4.8
ZnO	2.0

The pH was controlled between 6.9 and 7.2 by the addition of NaOH solution, and the temperature maintained at 30°C. IPTG (0.24g/l) was added after inoculation. After 24 hrs, the biomass had reached an OD of 34. Cells were then harvested by centrifugation and stored at -15°C, then used in biotransformations as required.

Example 2 **Deprotection of (D)-N-Acetyl-(1-bromovinyl)alanine**

KH_2PO_4 (0.8g, 10mmol) was dissolved in water (800 ml) in a 2 litre jacketed vessel. (D)-N-acetyl-(1-bromovinyl)alanine (100g, 0.42 mol, ~95% ee_R) was added and the pH adjusted to 8.0 using NaOH (46-48%). The temperature of the jacketed vessel was raised to 40°C and the solution stirred for 10 minutes while maintaining the pH at 8. The D-aminoacylase enzyme whole cells (9g) were added in one portion and the reaction mixture stirred at 40°C while maintaining the pH at 8.0 by subsequent additions of NaOH. The reaction was monitored by chiral GC as follows: 0.5 ml of the reaction mixture was taken and acidified to pH 2.0 with conc. HCl. The aqueous was extracted with EtOAc which was dried ($MgSO_4$) and filtered and treated with 0.1ml of TMS-diazomethane. The derivatised product was assayed by chiral GC (Chrompack Chirasil L-Val, 25m, 20psi He, 60°C for 10 mins, 5°C/min to 200°C, holding for 10 minutes, FID detection). After 1 hour the ee of the substrate had decreased to 68%, after 2 hours it was 24% and after 22 hours was 7%. The reaction mixture was then acidified to pH 2.0 using concentrated HCl solution, then filtered through a celite pad, and washed with EtOAc (3 x 300ml).

The solution was then adjusted to pH 6.5 using NaOH (46-48%) and concentrated under reduced pressure until about 200 ml of solution remained. A white solid crashed out of solution and was filtered off and washed with acetone. This gave the product (D)-(1-bromovinyl)alanine as a clean white solid (60g, ee_R>99 %). The enantiomeric excess was measured by HPLC (25cm Chirobiotic T column, 70:30 MeOH:water, 1ml/min, 210nm). Other aminoacids were determined by the same method or with variations in the MeOH:water mobile phase composition.

Example 3 **Deprotection of (D)-N-acetylpropargylglycine**

KH_2PO_4 (2.1g) was dissolved in water (2.5l) in a 2 litre jacketed vessel. The (D)-N-acetylpropargylglycine (232g, 1.50mol, ~95% ee_R) was added and the pH adjusted to 8.0 using NaOH (46-48%). The temperature of the jacketed vessel was raised to 40°C and the solution stirred for 10 minutes while maintaining the pH at 8. The D-aminoacylase enzyme whole cells (7g) were added in one portion and the reaction mixture stirred at

40°C while maintaining the pH at 8.0 by subsequent additions of NaOH. After 88 hours the remaining $ee_{(R)\text{-}N\text{-}Ac}$ was 25.8%, only marginally lower than that at 16 hours. Another 3%/wt of cells were added and after 104 hrs the $ee_{(R)\text{-}N\text{-}Ac}$ was 17.8%. The biotransformation was worked up since assuming 99% ee of product conversion was 85%.

Work-up and isolation as for Example 2 gave a brownish solid (271g) which was slurried in MeOH for 10 minutes to give a clean white solid (171g) of >99% ee (R)-propargylglycine.

Example 4 **Deprotection of (D)-N-acetyl-2-furylalanine**

(D)-N-Acetyl-2-furylalanine (18g, ee_R>99%) was added to water (200ml) containing 10mmole of KH_2PO_4 and the temperature of the jacketed vessel raised to 40°C. The suspension was then adjusted to pH 8.0 with NaOH and stirred for 10 minutes. 0.72g (4% wt) of D-aminoacylase whole cells were then added and the reaction stirred vigorously while maintaining the pH at 8.0 by subsequent addition of NaOH. The reaction was followed by chiral GC by the method described above, and was complete after 2 hours. The aqueous was the acidified to pH 2.0 with conc. HCl, and filtered through a Celite pad. The filtrate was then washed with EtOAc and adjusted to pH 7 with NaOH. The solution was the treated with Na_2CO_3 (2 equivalents assuming 100% conversion), cooled to ~10°C and then a solution of Fmoc-OSu (1 equivalent) in THF (300ml) added. After work-up the product was isolated as a white solid and recrystallised from methanol/water to give 18.4g of >99% ee (D)-2-furylalanine.

Example 5 **Deprotection of (D)-N-acetylallylglycine**

(D)-N-Acetylallylglycine (40g, ee_R 89%) was added to water (200ml) containing 10mmole of KH_2PO_4 and the temperature of the jacketed vessel raised to 40°C. The suspension was then adjusted to pH 8.0 with NaOH and stirred for 10 minutes. 1.0g (2.5% wt) of D-aminoacylase whole cells were then added and the reaction stirred vigorously while maintaining the pH at 8.0 by subsequent addition of NaOH. After 3 hours HPLC showed 30% conversion. After 16 hours the reaction had not gone any further and a further 2.5% wt of whole cells were added. After 60 hours the reaction still had not progressed past 30% and the solution was diluted to 10% by the addition of water. After another 2 hours, the reaction had reached 44% conversion, and after 88 hours 82% conversion.

Example 6 **Deprotection of (D)-N-acetyl-2-naphthylalanine**

(D)-N-Acetyl-2-naphthylalanine (0.75g) was added to 50ml Tris buffer (0.1 M, pH 7.5, 30 °C). The suspension was then adjusted to pH 8.0 with NaOH and stirred for 10 minutes. A crude lysate of D-aminoacylase (3 ml, 165U, 1U=hydrolysis of 1µmol of N-acetyl-D-tryptophan/min at 25°C, pH 7.5, 0.1M Tris buffer) was then added and the reaction stirred while maintaining the pH at 8.0 by subsequent addition of NaOH. More substrate (0.75g) and enzyme (165U) was added at 22, 46, 96, 173 and 218 hours. The final conversion by HPLC was 95% by peak area.

Example 7 **Deprotection of (D)-N-acetyl-3-pyridylalanine**

(D)-N-Acetyl-3-pyridylalanine (50g) was added to 750ml Tris buffer (0.1 M, pH 7.5, 30°C). The suspension was then adjusted to pH 8.0 with NaOH and stirred for 10 minutes. A crude lysate of D-aminoacylase (20 ml, 1100U, 1U=hydrolysis of 1µmol of N-acetyl-D-tryptophan/min at 25°C, pH 7.5, 0.1M Tris buffer) was then added and the reaction stirred while maintaining the pH at 8.0 by subsequent addition of NaOH. After 15 hours the reaction had reached about 80% conversion as measured by HPLC peak area.

Examples 8 to 20 **D-Acylase Reactions**

Table 1 reports D-acylase reactions using a range of unnatural (*R*)-N-Ac-phenylalanine and (*R*)-N-Ac-alanine derivatives, and (*R*)-N-Ac-4-fluorophenylglycine.

Table 1

Ex.	Substrate (N-Acetyl-)	Substrate Conc.		Enzyme (U/mmol)	pH[b]	Temp (°C)	Time[c]	Yield[d] (%)	Conv[e] (%)
		g/L	Mol/L						
8	4-Chloro-Phe-Ala	20	0.083	1.0[g]	8.0	40	72hrs	67	61
9	4-Bromo-Phe-Ala	30	0.104	50.2	8.6	40	>3days	73	90
10	4-Fluoro-Phe-Ala	84	0.373	26.5	7.5	30	4days	73	98
11	4-Cyano-Phe-Ala	15	0.069	18.5	7.7-8.0	30	>3days	45	75
12	2-Fluoro-Phe-Ala	100	0.440	2.2	8.0	40	24hrs	79	90
13	Styryl-Ala	13	0.056	160	8.5	30	3days	84	90
14	2-Thienyl-Ala	70	0.330	21.3	7.7	30	3days	56	95
15	5-Br-2-thienyl-Ala	44.4	0.152	14.6[g]	8.2	40	48hrs	86	>95
16	3-Thienyl-Ala	100	0.470	24.3	8.5	30	48hrs	87	94
17	3-Furyl-Ala	30	0.152	105	8.5	40	24hrs	n/a	90
18	2-Naphthyl-Ala	15	0.062	3.80[g]	8.5	40	7days	n/a	90
19	TAZ	112	0.525	69.1	7.5	30	3days	n/a	90
20	4-Fluoro-Phe-Gly	85.7	0.406	21.6	8.1	35	24hrs	88	n/a

[b] All reactions carried out in $NaOH/KH_2PO_4$ buffer. [c] Time to reach completion/cessation of reaction. [d] Isolated yield. [e] Conversion in crude biotransformation reaction mixture. [f]%e.e. rose from 90% in N-acetyl substrate to 96.5% in aminoacid. [g] Reaction done using whole cells.

TAZ = 4-ThiazoylAlanine

Comparative Testing

In order to evaluate the properties of the novel enzyme and known D-acylases, comparative experiments were run. The results are reported in Table 2. In each set of 3 results, the respective enzymes were those deposited as IFO 12806 (104470) and CCRC 14817 (104476), and that of Example 1 (D-Ace). The results (U/U) show that, when appropriate corrections have been made, the novel enzyme converts certain unnatural aminoacids, e.g. (R)-N-Ac-thienylalanine, faster than CCRC 14817, although the rate is slower for (R)-N-Ac-4-chlorophenylalanine.

Example 21 Whole Cell Immobilisation

Whole cells of *E. coli* CMC4406 containing recombinant D-acylase were immobilised on a reactive soluble polymer (RSP). The RSP was prepared by reaction of polyethyleneimine (0.8g) with aqueous 25% w/v glutaraldehyde (1.6ml), to a total volume of 20 ml H_2O. The RSP was then mixed with 10g of cells resuspended in 20ml H_2O. This was stirred vigorously for 30 minutes, after which the immobilised cells, having the consistency of foam rubber, were recovered by filtration. The final product (20g) had a specific activity of 20.55 U/g and the recovery of activity was 43% of the whole cells used in the immobilisation. 1 Unit of activity is defined as the hydrolysis of 1µmol/min N-Ac-D-tryptophan to D-tryptophan measured at a substrate concentration of 10mM at 25°C, pH7.5.

Example 22 Whole Cell Immobilisation

An aqueous suspension of cells of *E. coli* CMC4406 (10g in 20ml of water) was mixed thoroughly with 0.8g PEI, before the addition of 1.6ml of 25% w/v glutaraldehyde. Stirring of this mixture resulted in the formation of bead-like aggregates which were recovered by filtration. The final product (13.5g) had a specific activity of 20.39U/g and yielded 25% of the starting activity.

Table 2

Substrate	Enzyme	Dilution	Enz loading U/ml (N-Ac-DL-Trp)	Sub Loading (mM)	% Conversion	Time (Hrs)	Activity (U/U)	Activity (U/g)	Activity (U/ml)
(+/-)-N-Ac-4-Fluoro-Phe-Gly	104470	2	0.005	10	2	30	2	0	0
(+/-)-N-Ac-4-Fluoro-Phe-Gly	104476	2	0.115	10	22.4	30	1	2	0
(+/-)-N-Ac-4-Fluoro-Phe-Gly	D-Ace	200	0.2775	10	7.6	30	0	20	8
(R)-N-Ac-Thienyl Ala	104470	2	0.005	10	0.3	30	-	-	-
(R)-N-Ac-Thienyl Ala	104476	2	0.115	10	26.6	30	1	3	0
(R)-N-Ac-Thienyl Ala	D-Ace	200	0.2775	10	65	6	7	840	361
(R)-N-Ac-Naphthyl Ala	104470	2	0.005	50	1.5	22	11	1	0
(R)-N-Ac-Naphthyl Ala	104476	2	0.115	50	2.4	22	1	2	0
(R)-N-Ac-Naphthyl Ala	D-Ace	200	0.2775	50	1.7	22	0	30	13
(R)-N-Ac-Styryl Ala	104470	2	0.005	50	1.8	22	14	1	0
(R)-N-Ac-Styryl Ala	104476	2	0.115	50	1	22	-	-	-
(R)-N-Ac-Styryl Ala	D-Ace	200	0.2775	50	3.5	22	0	62	27
(R)-N-Ac-4-Chloro-Phe-Ala	104470	2	0.005	50	0.3	22	-	-	-
(R)-N-Ac-4-Chloro-Phe-Ala	104476	2	0.115	50	7.1	22	2	5	1
(R)-N-Ac-4-Chloro-Phe-Ala	D-Ace	200	0.2775	50	26	22	4	458	197
N-Ac-DL-Leu	104470	2	0.005	20	29	4	483	48	5
N-Ac-DL-Leu	104476	10	0.023	20	31	4	112	258	26
N-Ac-DL-Leu	D-Ace	5000	0.0111	20	13	4	98	12,597	5,417
N-Ac-DL-Met	104470	2	0.005	20	21	4	350	35	4
N-Ac-DL-Met	104476	10	0.023	20	17	4	62	142	14
N-Ac-DL-Met	D-Ace	5000	0.0111	20	7	4	53	6,873	2,917

Table 2 (contd.)

N-Ac-DL-Phe	104470	10	0.001	20	5	5.25	317	32	3
N-Ac-DL-Phe	104476	20	0.0115	20	22	5.25	121	279	28
N-Ac-DL-Phe	D-Ace	5000	0.0111	20	6	5.25	34	4,430	1,905
N-Ac-DL-Trp	104470	2	0.005	25	16	23.5	57	6	1
N-Ac-DL-Trp	104476	2	0.115	25	36	1	130	300	30
N-Ac-DL-Trp	D-Ace	5000	0.0111	25	13	2	244	31,492	13,542
N-Ac-DL-Val	104470	10	0.001	20	9	4	750	75	8
N-Ac-DL-Val	104476	10	0.023	20	38	4	138	317	32
N-Ac-DL-Val	D-Ace	1000	0.0555	20	4	4	6	775	333
N-Ac-D-Phe	104470	10	0.001	10	7	5.25	222	22	2
N-Ac-D-Phe	104476	20	0.0115	10	14	5.25	39	89	9
N-Ac-D-Phe	D-Ace	5000	0.0111	10	10	1	150	19,380	8,333
N-Ac-D-Trp	104470	2	0.005	10	20	23.5	28	3	0
N-Ac-D-Trp	104476	2	0.115	10	36	2	26	60	6
N-Ac-D-Trp	D-Ace	5000	0.0111	10	14	1	210	27,132	11,667
N-Benzoyl-DL-Phe	104470	10	0.001	20	0.1	5.25	-	-	-
N-Benzoyl-DL-Phe	104476	10	0.023	20	42	5.25	116	267	27
N-Benzoyl-DL-Phe	D-Ace	5000	0.0111	20	1.7	5.25	10	1,255	540

CLAIMS

1. An isolated enzyme capable of hydrolysing N-acetyl-D-tryptophan at a substrate concentration of 10 g/l and which exhibits faster conversion of (R)-N-acetyl-2-thienylalanine than of (R)-N-acetyl-4-chlorophenylalanine.

2. An isolated enzyme having the aminoacid sequence of SEQ ID NO: 4, or a fragment thereof capable of hydrolysing N-acetyl-D-tryptophan at a substrate concentration of 10 g/l.

3. An enzyme according to claim 1 or claim 2, wherein the substrate concentration is 30 g/l.

4. An enzyme according to claim 3, wherein the substrate concentration is 100 g/l.

5. An enzyme according to any preceding claim, in immobilised form.

6. An isolated polynucleotide encoding an enzyme according to claim 2.

7. A polynucleotide according to claim 6, having part or all of the sequence shown in SEQ ID No. 3.

8. A microorganism transformed to express an enzyme according to any of claims 1 to 5.

9. A microorganism having the characteristics of NCIMB 40965.

10. A method for producing an enzyme according to any of claims 1 to 5, which comprises culturing a microorganism according to claim 8 or claim 9.

11. A process for the preparation of a (D)-aminoacid, which comprises the conversion of a corresponding (D)-N-acylaminoacid using an enzyme according to any of claims 1 to 5 or a microorganism according to claim 8 or claim 9.

12. A process according to claim 11, wherein the concentration of the N-acylaminoacid is at least 30g/l.

13. A process according to claim 11, wherein the concentration of the N-acylaminoacid is at least 100 g/l.

14. A process according to any of claims 11 to 13, wherein the (D)-N-acylaminoacid is part of a mixture of the (L)- and (D)-N-acylaminoacids.

15. A process according to any of claims 11 to 13, wherein the (D)-N-acylaminoacid is enantiomerically enriched.

16. A process according to any of claims 11 to 13, wherein the (D)-N-aminoacid is essentially a single enantiomer.

17. A process according to claims 11 to 16, wherein the aminoacid is unnatural.

18. A process according to any of claims 11 to 15, wherein the substrate is hydrophobic, such that there is substrate inhibition, which comprises adding the substrate batchwise during the conversion.

19. A process according to any of claims 11 to 18, wherein the concentration of the accumulated D-aminoacid is at least 30 g/l.

SEQUENCE LISTING

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Arg Asp Met Thr Pro Lys Ile Ser Gln Gly Val Thr Thr Val Val Thr
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Phe Ala Asp Tyr Leu Asp Ala Leu Arg Ala Thr Pro Ala Ala Val Asn
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Ala Ala Cys Met Val Gly His Ser Thr Leu Arg Ala Ala Val Met Pro
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Ile Arg Arg Lys Leu Asp Val Pro Val Val Ile Ser His His Lys Val
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Arg Thr Ile Ile Thr Trp Cys Lys Pro Phe Pro Glu Leu Ser Gly Arg
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Val Val Phe Asp Pro Ala Thr Val Ala Asp Thr Ala Thr Phe Glu His
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Pro Thr Glu Arg Ala Ala Gly Ile His Ser Val Tyr Val Asn Gly Ala
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Asp Tyr Leu Asp Ala Leu Arg Ala Thr Pro Ala Ala Val Asn Ala Ala
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Arg Lys Leu Asp Val Pro Val Val Ile Ser His His Lys Val Met Gly
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Ala Gly Arg Gly Gln Leu Gln Ala Gly Tyr Phe Ala Asp Leu Val Val
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Phe Asp Pro Ala Thr Val Ala Asp Thr Ala Thr Phe Glu His Pro Thr
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INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/GB 99/03458

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/55 C12N9/80 C12N11/14 C12P41/00 C12P13/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M. WAKAYAMA ET AL.: "Cloning and sequencing of a gene encoding D-aminoacylase from Alcaligenes xylosoxydans subsp. sylososydans A-6 and expression of the gene in Escherichia coli." BIOSCIENCE, BIOTECHNOLOGY AND BIOCHEMISTRY, vol. 59, November 1995 (1995-11), pages 2115-2119, XP002105914 cited in the application the whole document — -/—	1-19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 December 1999	11/01/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Hix, R

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INTERNATIONAL SEARCH REPORT

Int. .tional Application No
PCT/GB 99/03458

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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Y	US 5 206 162 A (TSAI YING C ET AL) 27 April 1993 (1993-04-27) the whole document	1-19
Y,P	EP 0 896 057 A (DAICEL CHEM) 10 February 1999 (1999-02-10) the whole document	1-19
Y	M. WAKAYAMA ET AL.: "Overproduction of D-aminoacylase from Alcaligenes xylosoxydans subsp. xylosoxydans A-6 in Escherichia coli and its purification." PROTEIN EXPRESSION AND PURIFICATION, vol. 7, 1996, pages 395-399, XP002105916 cited in the application the whole document	1-19
A	M. SUGIE ET AL.: "Optical resolution of DL-amino acids with D-aminoacylase of Streptomyces." AGRICULTURAL & BIOLOGICAL CHEMISTRY, vol. 44, no. 5, 1980, pages 1089-1095, XP002105917 cited in the application the whole document	
A	EP 0 304 021 A (TAKEDA CHEMICAL INDUSTRIES LTD) 22 February 1989 (1989-02-22) table 3	
A	DE 24 19 838 A (AJINOMOTO KK) 21 November 1974 (1974-11-21) example 4	
A	DE 23 44 060 A (AJINOMOTO KK) 7 March 1974 (1974-03-07) example 4	
A	PATENT ABSTRACTS OF JAPAN vol. 014, no. 548 (C-0785), 5 December 1990 (1990-12-05) & JP 02 234677 A (DAI ICHI PURE CHEM CO LTD), 17 September 1990 (1990-09-17) abstract	

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INTERNATIONAL SEARCH REPORT

Inte. ional Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	PATENT ABSTRACTS OF JAPAN vol. 013, no. 174 (C-589), 25 April 1989 (1989-04-25) & JP 01 005488 A (DAICEL CHEM IND LTD), 10 January 1989 (1989-01-10) abstract	
A	PATENT ABSTRACTS OF JAPAN vol. 011, no. 353 (C-457), 18 November 1987 (1987-11-18) & JP 62 126976 A (AGENCY OF IND SCIENCE & TECHNOL;OTHERS: 02), 9 June 1987 (1987-06-09) abstract	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/GB 99/03458

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
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EP 0896057	A	10-02-1999	JP	11098982 A	13-04-1999
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			CA	1018920 A	11-10-1977
			CH	577438 A	15-07-1976
			FR	2197836 A	29-03-1974
			GB	1429976 A	31-03-1976
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JP 02234677	A	17-09-1990	JP	2869793 B	10-03-1999
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Ala Ala Cys Met Val Gly His Ser Thr Leu Arg Ala Ala Val Met Pro
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gac ttg cag cgc gcc gcc acc gac gag gaa atc gcg gcc atg cgg gac     528
Asp Leu Gln Arg Ala Ala Thr Asp Glu Glu Ile Ala Ala Met Arg Asp
    160                 165                 170
```

```
ctg gcc gag gaa gcc atg gcc agc ggc gcc atc ggc att tcg acc ggc   576
Leu Ala Glu Glu Ala Met Ala Ser Gly Ala Ile Gly Ile Ser Thr Gly
175                 180                 185                 190

gcc ttc tac ccg ccc gcc gcc cgc gcc acc acc gaa gag atc atc gag   624
Ala Phe Tyr Pro Pro Ala Ala Arg Ala Thr Thr Glu Glu Ile Ile Glu
                195                 200                 205

gtg tgc cgg ccg ctg agc gcg cat ggc ggc atc tac gcc acc cac atg   672
Val Cys Arg Pro Leu Ser Ala His Gly Gly Ile Tyr Ala Thr His Met
            210                 215                 220

cgc gac gaa cgc gag cac atc gtg gcc gcg ctg gag gaa acc ttc cgc   720
Arg Asp Glu Arg Glu His Ile Val Ala Ala Leu Glu Glu Thr Phe Arg
        225                 230                 235

atc cgc cgc aag ctg gac gtg ccg gtg gtg atc tcg cac cac aag gtc   768
Ile Arg Arg Lys Leu Asp Val Pro Val Val Ile Ser His His Lys Val
    240                 245                 250

atg ggc cag ccc aat ttc ggc cgc tcg cgc gag acg ctg ccg ctg atc   816
Met Gly Gln Pro Asn Phe Gly Arg Ser Arg Glu Thr Leu Pro Leu Ile
255                 260                 265                 270

gag gcc gcc atg gcg cgc cag gac gtc tcg ctg gac gcg tat ccc tac   864
Glu Ala Ala Met Ala Arg Gln Asp Val Ser Leu Asp Ala Tyr Pro Tyr
                275                 280                 285

gtg gcc ggc tcc acc atg ctc aag cag gac cgc gtg ctg ctg gcc ggg   912
Val Ala Gly Ser Thr Met Leu Lys Gln Asp Arg Val Leu Leu Ala Gly
            290                 295                 300

cgc acc atc atc acc tgg tgc aag ccc ttc ccc gaa ctg agc ggg cgc   960
Arg Thr Ile Ile Thr Trp Cys Lys Pro Phe Pro Glu Leu Ser Gly Arg
        305                 310                 315

gac ctg gat gaa gtc gcg gcc gag cgc ggc aaa tcc aag tac gac gtg   1008
Asp Leu Asp Glu Val Ala Ala Glu Arg Gly Lys Ser Lys Tyr Asp Val
    320                 325                 330

gtg ccc gag ctg cag ccg gcc ggc gcc atc tac ttc atg atg gac gaa   1056
Val Pro Glu Leu Gln Pro Ala Gly Ala Ile Tyr Phe Met Met Asp Glu
335                 340                 345                 350

ccc gac gtg cag cgc atc ctg gcg ttc ggc ccg acc atg atc ggc tcc   1104
Pro Asp Val Gln Arg Ile Leu Ala Phe Gly Pro Thr Met Ile Gly Ser
                355                 360                 365
```

```
gac ggc ctg ccg cac gac gag cgc ccg cat ccg cgc ctg tgg ggc acc   1152
Asp Gly Leu Pro His Asp Glu Arg Pro His Pro Arg Leu Trp Gly Thr
            370                 375                 380

ttc ccg cgg gtg ctg ggg cac tat gcg cgc gac ctg ggc ctg ttc ccg   1200
Phe Pro Arg Val Leu Gly His Tyr Ala Arg Asp Leu Gly Leu Phe Pro
        385                 390                 395

ctg gag acg gcg gta tgg aag atg acc ggc ctg acc gcc gcg cgc ttc   1248
Leu Glu Thr Ala Val Trp Lys Met Thr Gly Leu Thr Ala Ala Arg Phe
    400                 405                 410

ggc ctg gcc ggg cgc ggg cag ctg cag gcc ggg tac ttc gcc gac ctg   1296
Gly Leu Ala Gly Arg Gly Gln Leu Gln Ala Gly Tyr Phe Ala Asp Leu
415                 420                 425                 430

gtg gtg ttc gac ccg gcc acg gtg gcc gat acc gcc acc ttc gaa cac   1344
Val Val Phe Asp Pro Ala Thr Val Ala Asp Thr Ala Thr Phe Glu His
                435                 440                 445

cct acc gag cgc gcc gcc ggc atc cat tcc gtg tac gtc aac ggc gcg   1392
Pro Thr Glu Arg Ala Ala Gly Ile His Ser Val Tyr Val Asn Gly Ala
            450                 455                 460

ccg gtc tgg caa gag cag gcg ttc acc ggc cag cat gcc ggc cgc gtg   1440
Pro Val Trp Gln Glu Gln Ala Phe Thr Gly Gln His Ala Gly Arg Val
        465                 470                 475

ctc gca cgc acg gcc gcc tgagctcgaa ttcac                          1479
Leu Ala Arg Thr Ala Ala
    480
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<212> PRT
<213> Alcaligenes

<400> 4

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Met Ala Glu Ser Asp Ser Gln Pro Phe Asp Leu Leu Leu Val Gly Gly
  1               5                  10                  15

Thr Leu Ile Asp Gly Ser Asn Thr Pro Gly Arg Arg Ala Asp Leu Gly
            20                  25                  30

Val Arg Gly Asp Arg Ile Ala Ala Ile Gly Asp Leu Ser Asp Ala Ala
        35                  40                  45
```

Ala His Thr Arg Val Asp Val Ser Gly Leu Val Val Ala Pro Gly Phe
50 55 60

Ile Asp Ser His Thr His Asp Asp Asn Tyr Leu Leu Arg Arg Arg Asp
65 70 75 80

Met Thr Pro Lys Ile Ser Gln Gly Val Thr Thr Val Val Thr Gly Asn
85 90 95

Cys Gly Ile Ser Leu Ala Pro Leu Ala His Ala Asn Pro Pro Ala Pro
100 105 110

Leu Asp Leu Leu Asp Glu Gly Gly Ser Tyr Arg Phe Glu Arg Phe Ala
115 120 125

Asp Tyr Leu Asp Ala Leu Arg Ala Thr Pro Ala Ala Val Asn Ala Ala
130 135 140

Cys Met Val Gly His Ser Thr Leu Arg Ala Ala Val Met Pro Asp Leu
145 150 155 160

Gln Arg Ala Ala Thr Asp Glu Glu Ile Ala Ala Met Arg Asp Leu Ala
165 170 175

Glu Glu Ala Met Ala Ser Gly Ala Ile Gly Ile Ser Thr Gly Ala Phe
180 185 190

Tyr Pro Pro Ala Ala Arg Ala Thr Thr Glu Glu Ile Ile Glu Val Cys
195 200 205

Arg Pro Leu Ser Ala His Gly Gly Ile Tyr Ala Thr His Met Arg Asp
210 215 220

Glu Arg Glu His Ile Val Ala Ala Leu Glu Glu Thr Phe Arg Ile Arg
225 230 235 240

Arg Lys Leu Asp Val Pro Val Val Ile Ser His His Lys Val Met Gly
245 250 255

Gln Pro Asn Phe Gly Arg Ser Arg Glu Thr Leu Pro Leu Ile Glu Ala
260 265 270

Ala Met Ala Arg Gln Asp Val Ser Leu Asp Ala Tyr Pro Tyr Val Ala
275 280 285

Gly Ser Thr Met Leu Lys Gln Asp Arg Val Leu Leu Ala Gly Arg Thr
290 295 300

```
Ile Ile Thr Trp Cys Lys Pro Phe Pro Glu Leu Ser Gly Arg Asp Leu
305                 310                 315                 320

Asp Glu Val Ala Ala Glu Arg Gly Lys Ser Lys Tyr Asp Val Val Pro
                325                 330                 335

Glu Leu Gln Pro Ala Gly Ala Ile Tyr Phe Met Met Asp Glu Pro Asp
            340                 345                 350

Val Gln Arg Ile Leu Ala Phe Gly Pro Thr Met Ile Gly Ser Asp Gly
        355                 360                 365

Leu Pro His Asp Glu Arg Pro His Pro Arg Leu Trp Gly Thr Phe Pro
    370                 375                 380

Arg Val Leu Gly His Tyr Ala Arg Asp Leu Gly Leu Phe Pro Leu Glu
385                 390                 395                 400

Thr Ala Val Trp Lys Met Thr Gly Leu Thr Ala Ala Arg Phe Gly Leu
                405                 410                 415

Ala Gly Arg Gly Gln Leu Gln Ala Gly Tyr Phe Ala Asp Leu Val Val
            420                 425                 430

Phe Asp Pro Ala Thr Val Ala Asp Thr Ala Thr Phe Glu His Pro Thr
        435                 440                 445

Glu Arg Ala Ala Gly Ile His Ser Val Tyr Val Asn Gly Ala Pro Val
    450                 455                 460

Trp Gln Glu Gln Ala Phe Thr Gly Gln His Ala Gly Arg Val Leu Ala
465                 470                 475                 480

Arg Thr Ala Ala
```